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EVALUATION OF INHIBITORY MEASURES FOR FOOD SPOILER YEAST *CANDIDA KRUSEI* DURING FERMENTATION PROCESS BY CHEMICAL, BIOCHEMICAL AND NANOPARTICLE APPROACHES

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ABSTRACT

Screening of chemical, biochemical and biomolecule-nanoparticle methods for the inhibition of *Candida krusei* were evaluated without hampering the growth of dairy yeast *Kluyveromyces marxianus*. The effective inhibition was observed with the help of H_2O_2, *Williopsis saturnus*, at specific combination of pH and temperature (pH 5.0 and 40 °C) and Ag-KT4561 nanoparticles among the various methods used. However, the most efficient inhibition was observed with Ag-KT4561 nanoparticles. In general H_2O_2 works best at pH range 4.0 to 10.0 and at temperature 30 °C or above. H_2O_2 concentration of 4000 ppm at 45 °C and pH 5.5 exhibited significant inhibition of *C. krusei*, while *K. marxianus* remains unaffected. But, when used with lyophilized supernatant of *W. saturnus*, 2400 ppm H_2O_2 was effective. Further, nanoparticle with silver was synthesized to reduce the quantity of killer protein and enhance the efficiency of protein. Complete inhibition of *C. krusei* was observed at 350 μM of synthesized silver nano-particle (AgNPs) of the killer protein from *W. saturnus*, with little effect on *K. marxianus* concentration. A stability test confirms the effect of protein silver nanoparticles on *C. krusei* for more than 20 weeks without any change in pH and temperature. Thus, the nanoparticles could be potentially used for inhibition of *C. krusei* without affecting the growth of *K. marxianus* and the process could be run non-aseptically.

Keywords: H_2O_2, Spoiler yeast, Inhibition, Killer protein, Ag-KT4561 bio-molecule nanoparticles, Green chemistry

INTRODUCTION

Cheese whey (a byproduct of cheese processing industries) has been efficiently exploited for the production of single cell protein (SCP) over the years as cheese whey has an immense nutritional value **(Ayoola *et al.*, 2008; Carvalho *et al.*, 2013)**. It contains 4.5-5% (w/v) of lactose, 0.6-0.8% (w/v) of soluble proteins, 0.4-0.5% (w/v) of lipids and 8-10% (w/v) of mineral salts of the dried extract. Efficient utilization of cheese whey for SCP conversion reduces the biochemical oxygen demand (BOD) by 75% and thus decreases the disposal problem **(Eyster, 1950; Prazeres *et al.*, 2012)**. Mostly lactose-consuming organisms, such as *Kluyveroymyces* spp. and *Lactobacillus* spp. grow in cheese whey **(Koleva *et al.*, 2008; Orru *et al.*, 2010)**. Production of SCP from cheese whey serves dual purpose by reducing environmental pollution and generating a valuable product (i.e. proteinaceous biomass which is used as animal feed and food ingredients) **(Koleva *et al.*, 2008)**. Likewise, *Kluyveroymyces marxianus* has been grown in cheese whey as a mono-culture for SCP production **(Yadav *et al.*, 2012)**. However, on a large scale industrial process, contamination is a major problem for SCP production despite the treatment of large volume of cheese whey. To eliminate this problem, certain extreme fermentation parameters have been applied, such as low pH (3.0-4.0) and high temperature (40-45 °C). At these conditions, most of the pathogenic microorganisms cannot survive **(Maneesri and Maneesri, 2009; Ahariz *et al.*, 2010)** and therefore a safe food or feed product is ensured during fermentation process. Additionally, the extreme fermentation conditions help to make the process economical due to reduced operating cost of maintaining sterility.

However, certain food spoilers (contaminant) e.g. *Candida krusei* still survive under extreme conditions (pH 3.0 and 45 °C) **(Guo and Bhattacharjee, 2006)**. The opportunistic *Candida* species exist as commensal in healthy individuals **(Heard and Fleet, 1988)**. During the production of SCP, *C. krusei* emerges as a contaminant while it grows along with *K. marxianus* and this is a concern for food safety. *C. krusei* is known as a food contaminant and an opportunistic pathogen **(Siso, 1996; Hornbæk *et al.*, 2006; Maneesri and Maneesri, 2007; Kim and Lee, 2012)**. However, *C. krusei* is reported to be present in many dairy and fermented food products, but yet does not come under generally recognized as safe (GRAS) microorganism **(Walker and Dijck, 2006; Walker *et al.*, 2008)**. Therefore, the eradication of *C. krusei* is essential from food products to meet safety regulations.

Certain chemical and biochemical approaches were reported to employ for selective inhibition of *C. krusei*. The chemical (NaCl, H_2O_2) and the biological inhibitors (medicinal plants, such as *Lupinus angustifolis*, *Syzigium aromaticum* (clove) oil; nisin and *Williopsis saturnus* and synergistic effect of *W. saturnus* and H_2O_2) to inhibit *C. krusei* have been reported **(Ayoola *et al.*, 2008; Dingman, 2008; Adeniyi *et al.*, 2010; Da Silva *et al.*, 2011)**. It was reported that H_2O_2 inhibited *C. krusei* **(Morgulis *et al.*, 1926)**. Apparently, *C. krusei* has also been tested against a wide range of essential oils where ethanol 70% v/v served as control **(Nel *et al.*, 2006; Souza *et al.*, 2008; Waema *et al.*, 2009)**. Another significant approach to inhibit *C. krusei* was using NaCl; however it depends on the sensitivity of the organism and the concentration of NaCl used. The sensitive strain of *C. krusei* undergoes cell death at 2 M concentration of NaCl **(Aguiar and Lucas, 2000)**.

Yeasts such as *Aspergillus furnigatus*, *W. saturnus* (major yeast from yogurt) have the capacity to produce killer proteins **(Fang *et al.*, 2002; Brock, 2008)**. The mycotoxins/killer proteins produced by *W. saturnus* have a broad spectrum of inhibitory activity at wide range of pH and temperature **(Buzzini *et al.*, 2004)**. These could be used as the versatile anti-spoilage agents for food and feed production **(Kao *et al.*, 1999; Liu *et al.*, 2006)**. Another killer protein is nisin, which is used for food preservation and is produced by *Lactobacillus* spp. or lactic acid bacteria (LAB) **(Guwy *et al.*, 1999)**. Nisin is used to stabilize food products and is often added to the cheese for inhibiting toxin production by *Clostridium botulinum*. It was also reported to inhibit *C. krusei* efficiently **(Lowes *et al.*, 2000; Russell and Jarvis, 2001)**.

In certain industrial fermentation processes, stress of pH shock was encountered to inhibit certain food spoiler yeasts **(Siso, 1996; Pinheiro *et al.*, 2002)**. At pH 2.0, *C. krusei* did not grow well **(Lowes *et al.*, 2000)**. In a mixed culture, where the presence of other yeast strains was also reported, effective utilization of any inhibitor (i.e. chemical or biochemical inhibitor against *C. krusei*) depends entirely upon whether the other type of yeast was also inhibited by the specific inhibitor being used. Another effective way of inhibition of pathogens is by the usage of metal nanoparticles (NPs) or biomolecule based nanoparticles **(Dingman, 2008)**. It has already been observed that silver NPs can kill pathogens

at very low concentrations and biomolecule based nanoparticles do not exert any toxic effects on human cells. Apart from that, silver NPs do not cause any microbial resistance and also there is no specific site of action for inhibition of the microbial cells **(Panacek *et al*., 2009)**. Hence, the aim of the present study was to evaluate different inhibition methods to inhibit *C. krusei* alone as well as in a mixed culture system without affecting the growth of *K. marxianus*.

MATERIALS AND METHODS

Chemicals

Analytical grade chemicals were used in the experiments. NaCl (Quelab Lab Inc., Montréal, Canada), H_2O_2 (Laboratoire Mat, Québec), yeast Extract (Fisher Scientific, USA), malt extract (Oxoid Ltd., Basingstoke, England), meat peptone (Organotechnie SA., La Courneuve, France), glucose, ethyl alcohol 95% (Fisher Scientific, USA), agar (Quebact Lab Inc., Montréal, Canada), cheese whey (Agropur, Canada), and $AgNO_3$ (Fisher Scientific, Ottawa).

Microorganisms

K. marxianus strain used in the study was isolated and characterized from the SCP production plant using cheese whey as substrate. *C. krusei* strain was also isolated and identified as a contaminant during SCP production employing cheese whey. *W. saturnus* strain DBVPG 4561 was obtained from the Industrial Yeasts Collection DBVPG of Perugia (Italy). Strains were sub-cultured on YEPD (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) agar slants and stored at 4 ^oC for further use.

Inhibition studies for *C. krusei*

Chemical Methods

Inhibition by NaCl

Pre-culture broth of *K. marxianus* of $2.0x10^8$ CFU/mL and *C. krusei* of $4.0x10^7$ CFU/mL were prepared in 100 mL YEPD media in 500 mL Erlenmeyer flasks. The medium pH was adjusted to 3.5 and sterilized at 121 ^oC for 15 min. After sterilization, 1.5 M and 2 M NaCl were added in different sets of flasks. *C. krusei* is a non-lactose assimilating organism, while *K. marxianus* is a lactose assimilating organism. Therefore, sterilized YEPD media were inoculated with 30 µL (from stock culture) of *K. marxianus* and 50 µL of *C. krusei*. Inoculated flasks were incubated at 40 °C for 24 h. Samples were taken at regular intervals for the analysis of total cell count. Total cell concentration was measured using standard agar plate technique **(Logothetis *et al*., 2007; Goretti *et al*., 2009;Kosseva *et al*., 2009)**.

Inhibition by H_2O_2

A pre-culture was prepared for *C. krusei* and *K. marxianus* as above. After that, culture media of cheese whey powder 4.5% (w/v) and urea 0.22% (w/v) were prepared and pasteurized at 80 °C for 20 min. The pasteurized cheese whey culture media at different pH (3.5, 4.5, 5.5, and 6.0) was added to different 500 mL flasks and inoculated with 1% (v/v) inoculum of *C. krusei* and *K. marxianus*. Different concentrations of H_2O_2 (100, 200, 300 and 400 ppm) were added to these flasks. After inoculation, flasks were incubated at 28 °C and 40 °C in an orbital incubator shaker for 24 h. Samples were drawn at regular intervals to analyze the total cell count.

Simultaneously, two different set of experiments were conducted, where in the primary set of experiments the H_2O_2 concentration were varied (0, 300, 400, 500, 600 and 800 ppm) and applied directly on the fermenter broth containing *K. marxianus* and *C. krusei*, which was collected from commercial continuous SCP production plant. 100 mL of fermented broth of *K. marxinaus* ($3.0x10^6$ CFU/mL) severely contaminated with *C. krusei* ($1.8x10^6$ CFU/mL) was taken in 500 mL 2 sterilized flasks. Flasks were incubated at pH 3.5, 150 rpm and 40 °C in an incubator shaker.

In the secondary set of experiments, variation in H_2O_2 concentration (2400, 3200 and 4000 ppm) were considered and applied directly to the fermenter broth and flasks were incubated at pH 5.0, 150 rpm and 45 °C in an incubator shaker.

Biochemical Methods

Inhibition by S. aromaticum oil

A set of experiments were conducted in which 0.4% (v/v) of clove oil was added in fermenter broth which contains *C. krusei* and *K. marxianus*. The initial cell count of *C. krusei* and *K. marxianus* was $5.0x10^6$ CFU/mL and $6.0x10^6$ CFU/mL, respectively. The flasks were placed in an orbital incubator shaker at 28 °C at 150 rpm for 6 h. Sampling was performed at an interval of 2 h. Samples were analyzed for total cell count using standard agar plate technique.

Inhibition with nisin

The culture of *C. krusei* and *K. marxianus* were grown separately in MRS broth at 35 ^oC for 24 h. Bioassay MRS media with 0.75% of Bacto agar and 1% Tween-20 were prepared. Media were sterilized at 121 ^oC for 15 min. A solution of nisin (1,000 IU/mL) was prepared by adding 0.025 g of commercial nisin (Sigma-Aldrich, Milwaukee, USA) into 25 ml of sterile solution of 0.02 N HCl. Sterilized media were cooled down to 40 ^oC and inoculated with 1% (v/v) of the 24 h culture of *C. krusei and K. marxianus* in two sets (duplicate). Then the bioassay agar (25 mL) was aseptically poured into sterile petri dishes (100x15 mm) and allowed to solidify for 1 h. On each plate, four or five holes were bored, using a 7 mm outer diameter stainless steel borer with a slight suction. An aliquot (50 μL and 100 μL) of standard nisin solution was placed into a well and the bioassay agar plate was incubated right away at 35 ^oC for 24 h. The control for each plate was prepared using sterile distilled water in wells. Zone of inhibition was observed in control and test samples.

Inhibition study with *W. saturnus*

Preparation of W. saturnus culture broth

YEPD (100 mL) was prepared in 500 mL flasks and sterilized at 121 ^oC for 15 min. The sterilized flask was inoculated with loopful of *W. saturnus* and incubated in an orbital incubator shaker at 150 rpm and 28 ^oC for 48 h. Samples were taken at regular time intervals for total cell count.

Well assay method

Pre-culture of *C. krusei* was prepared in YEPD as described above using 1% (v/v) inoculum. After 24 h, *C. krusei* sample was diluted 10^2, 10^3 and 10^4 times in saline solution and different diluted samples were spread plated in YEPD agar plates. After spread plating, wells were made in agar plates using borer and 60 μL of *W. saturnus* (48 h) culture was added in each well. The plates were incubated in an orbital incubator at 28 ^oC for 24 h. The plates were visually observed after 24 h.

To differentiate the morphology of *C. krusei* from *W. saturnus*, Methylene Blue Citric-Phosphate agar (MBA) plates were prepared and spread plated using *C. krusei* and *W. saturnus*. Plates were incubated for 24 h at 28 ^oC and were visually examined to check the morphology.

Inhibition by W. saturnus

W. saturnus was grown in YEPD and cheese whey medium for 24 h. YEPD and cheese whey powder 4.5% (w/v) with 0.22% (w/v) urea were prepared in two flasks of 2 L capacity each containing 500 mL medium. After sterilization, each flask was inoculated with 2% (v/v) *W. saturnus* and incubated in an orbital incubator shaker at 150 rpm and 28 °C. The culture was harvested at 48 h. The culture broth was centrifuged at 10 000 x g and the supernatant was lyophilized to obtain the powder which contained extracellular proteins. The extracellular proteins specifically contain a particular protein KT4561 (~ 62 kDa protein), which has anti-mycotic activity **(Buzzini *et al*., 2004)**. Simultaneously, another set of flasks containing *W. saturnus* were grown, where no centrifugation was performed. Henceforth, the cultures were directly taken for lyophilization.

Pre-cultures were prepared by growing *C. krusei*, *K. marxianus* and *W. saturnus* in YEPD medium for 24 h. One hundred milliliters of fresh cheese whey powder 4.5% (w/v) with urea 0.22% (w/v) solution was added to each five hundred milliliters Erlenmeyer flask (two flasks) and pasteurized at 80 °C for 20 min. After pasteurization, media were aseptically adjusted to different pH (3.5 and 4.5) followed by inoculation with 1% (v/v) mixed culture (*C. krusei* and *K. marxianus*).

Inhibition by lyophilized supernatant from W. saturnus

Various concentrations of lyophilized supernatant of *W. saturnus* were considered (well plate assay method) and the zone was created by the inhibitory effect of the killer protein. The inhibition zones were measured after 24 h of incubation at 30 ºC. A linear equation (y = 0.30x-0.36) was sketched out between the diameter of the clear zone (measured in millimeters, x axis) and the logarithm of the quantity of the killer protein (measured in nanograms, y axis). This method was used to determine the killer protein concentration required for the inhibition of *C. krusei* which is similar to the technique mentioned in **(Chen *et al*., 2000)**. Lyophilized supernatant prepared in YEPD media was served as the control and lyophilized cheese whey was the experimental product.

Inhibition of C. krusei by synergistic effect of H_2O_2 and W. saturnus

To study inhibition of *C. krusei*, different H_2O_2 concentrations were used along with *W. saturnus* (entire organism lyophilized supernatant powder, as described

above). 300 ppm of H_2O_2 was used along with 1% (v/v) of *W. saturnus* (inoculum from pre-culture) for the inhibition of *C. krusei* in a mixed culture of *C. krusei* and *K. marxianus* at pH 6.0 and 28 °C. Similar sets of experiments were conducted with a variation in pH (3.5-4.5) at 28 °C.

Two different set of experiments were conducted, where in the primary set of experiments was conducted where lyophilized *W. saturnus* was used by varying the H_2O_2 concentration directly on the fermenter broth containing *K. marxianus* and *C. krusei*, collected from commercial continuous SCP production plant. 100 mL of fermented broth of *K. marxinaus* ($3.1x10^6$ CFU/mL) grossly contaminated with *C. krusei* ($1.5x10^6$ CFU/mL) was taken in 500 mL 2 sterilized flasks. The lyophilized powder of *W. saturnus* (200 mg/mL) along with different concentrations of H_2O_2 (2400 and 4000 ppm) was then added to each flask. Flasks were incubated at pH 5.0, 150 rpm and 40 °C in an incubator shaker.

Whereas in secondary set of experiments, about 400 mg/mL of lyophilized supernatant of *W. saturnus* was used along with 2400 ppm of H_2O_2 in a mixed culture by adjusting the pH of fermenter broth to 5.0. As the killer protein produced by lyophilized *W. saturnus* is highly effective at pH range of 4.5-10.0 and temperature from 25 to 45 ºC **(Goretti *et al*., 2009)**. Flasks were kept at 150 rpm and 40 °C in an orbital incubator shaker.

Inhibition of *C. krusei* by Ag-KT4561 NPs

Synthesis of nanoparticles (Ag-KT4561) was carried out in the previous study **(Bhattacharya *et al*., 2015)**. However a bulk preparation of the same has been conducted in this study. During the scale-up process, 20 mL of 0.1 M $AgNO_3$ solution is continuously stirred along with 18 mL of *W. saturnus* supernatant at 25 °C for 48 h. Ag^+ ions were completely reduced at 48 h of stirring. After which the bulk nanoparticle solution was taken for lyophilization and the lyophilized product was tried against *C. krusei* in 4.5% (w/v) cheese whey and 0.22% (w/v) of urea. From the lyophilized product different concentration of Ag-KT4561 ranging from 10 µM - 1 mM were tried at pH 5.5 and 30 °C in shake flasks. In these experiments, mixed culture of *C. krusei* (2% (v/v)) and *K. marxianus* (2% (v/v)) were tested for 12 h and total cell concentration (CFU/mL) was measured at 3 h time intervals.

Analytical methods

Cell count

Total cell count as CFU (colony forming units) was estimated by standard agar plate technique in YEPD agar plates **(Nathan *et al*., 1978)**. The appropriately diluted samples were plated on agar plates and incubated at 30 ºC overnight to form fully developed colonies. The colonies of *K. marxianus*, *C. krusei* and *W. saturnus* were identified based on its morphology by visible examination.

Protein estimation

The soluble protein concentration was determined by Lowry's method **(Lowry *et al*., 1951)** using bovine serum albumin as standard.

UV-Vis Spectroscopy

The bulk sample of $AgNO_3$ and *W. saturnus* supernatant were prepared at 48 h and samples were collected at every 6 h to analyze for nanoparticle formation at 300-700 nm in Spectrophotometer (Carry 100 Bio®, Varian USA).

Statistical method

For each set, samples were analyzed in triplicates and mean values are taken in account. Further standard deviation of the colonies in log units (Log_{10} CFU/mL) were calculated for each experimental point in Microsoft excel 2013 external package similar to the method of **De Oliveira *et al*., 2014**.

RESULTS

Inhibition by NaCl

Different concentrations (1.5- 2 M) of NaCl were tested to inhibit the growth of *C. krusei* in mono-culture and the results were presented in Figure 1. It was clear that NaCl concentration of 2 M showed significant inhibition of *C. krusei* compared to 1.5 M without having any effect on *K. maxianus*. This is due to the fact that *C. krusei* exhibited salt-stress **(Aguiar and Lucas, 2000)** and got killed at pH 3.5 and 40 °C. No inhibition of *C. krusei* was observed when NaCl concentration was less than 2 M at pH 3.5 and 40 °C.

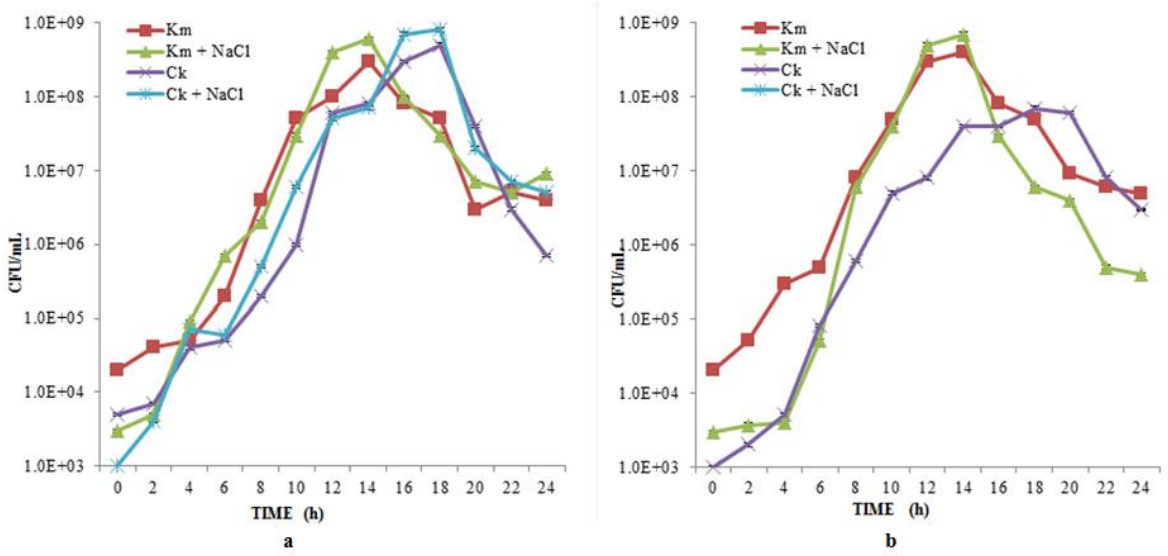

Figure 1 Impact of **a)**1.5 M NaCl and **b)** 2.0 M on *C. krusei* (Ck) and *K. marxinaus* (Km) in YEPD medium at pH 3.5 and 40 °C (Shake flask experiments)

Inhibition by H_2O_2

Viability of individual cultures of *C. krusei*, and *K. marxianus* at different concentrations of H_2O_2 (100 - 400 ppm) in cheese whey at pH 6.0 and 28 °C was studied (Table 1). It showed that *C. krusei* was not inhibited at lower concentration of H_2O_2. However, inhibition occurred at 300 ppm H_2O_2. *K. marxianus* was not inhibited at these concentrations of H_2O_2.

Table 1 Impact of various concentrations of H_2O_2 on *C. krusei* and *K. marxianus* in cheese whey powder at pH 6.0 and temperature 28 °C (Shake flask)

	Individual Organisms **(Log $_{10}$ CFU/mL) ± Standard Deviation**						
H_2O_2 (ppm)	**0**		**100**	**200**	**300**	**400**	
Time (h)	**Ck**	**Km**	**Ck**	**Ck**	**Ck**	**Ck**	**Km**
0	5.73±.07	4.83±.01	5.81±.01	5.81±.01	5.92±.01	5.15±.03	5.81±.02
12	6.52±.02	9.33±.01	6.08±.03	5.23±.01	5.40±.04	NG	9.26±.02
24	8.51±.02	8.31±.01	6.12±.05	5.18±.05	NG	NG	8.28±.02

Legend: NG- No Growth Observed, Ck – *C. krusei*, Km – *K. marxianus*

The inhibition of *C. krusei* in mixed cultures (*C. krusei* and *K. marxianus*) in cheese whey was studied and the results were presented in Figure 2. The concentration of H_2O_2 used was 300 ppm and 400 ppm (from previous results in Table 1). *C. krusei* was not inhibited at 300 - 400 ppm of H_2O_2. On the contrary, *C. krusei* dominated over *K. marxianus* in a mixed culture at 24 h.

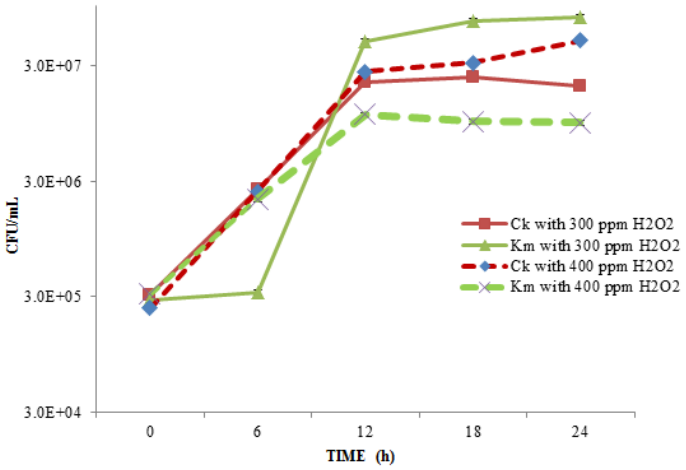

Figure 2 Impact of H_2O_2 on *C. krusei* (Ck), *K. marxianus* (Km) in cheese whey at pH 6.0 and 28 °C with 300 ppm and 400 ppm H_2O_2

Variations in pH along with 300 ppm H_2O_2 concentration

pH variations were carried out and it was lowered to 3.5 from 6.0 and studies of mixed culture (*C. krusei*, *K. marxianus* and *W. saturnus* 1% and 2% (v/v)) were also carried out maintaining similar parameters in cheese whey. *C. krusei* was not inhibited at these parameters, whereas growth of *K. marxianus* and *W. saturnus* remains unchanged.

A mixed culture study at pH 4.5 and 28 °C in cheese whey powder with 300 ppm H_2O_2 exhibited a partial inhibition of *C. krusei* (Figure 3a). However when bio-preservative *W. saturnus* was added at the similar condition, the growth of *C. krusei* decreased by one log-unit at 12 h (Figure 3b), but the growth accelerated after 12 h. As *W. saturnus* produces killer protein (KT4561) but the concentration of the killer protein remains low which is insufficient to inhibit *C. krusei* in a large-scale fermentation. Hence, this parameter could be considered for the inhibition of *C. krusei*.

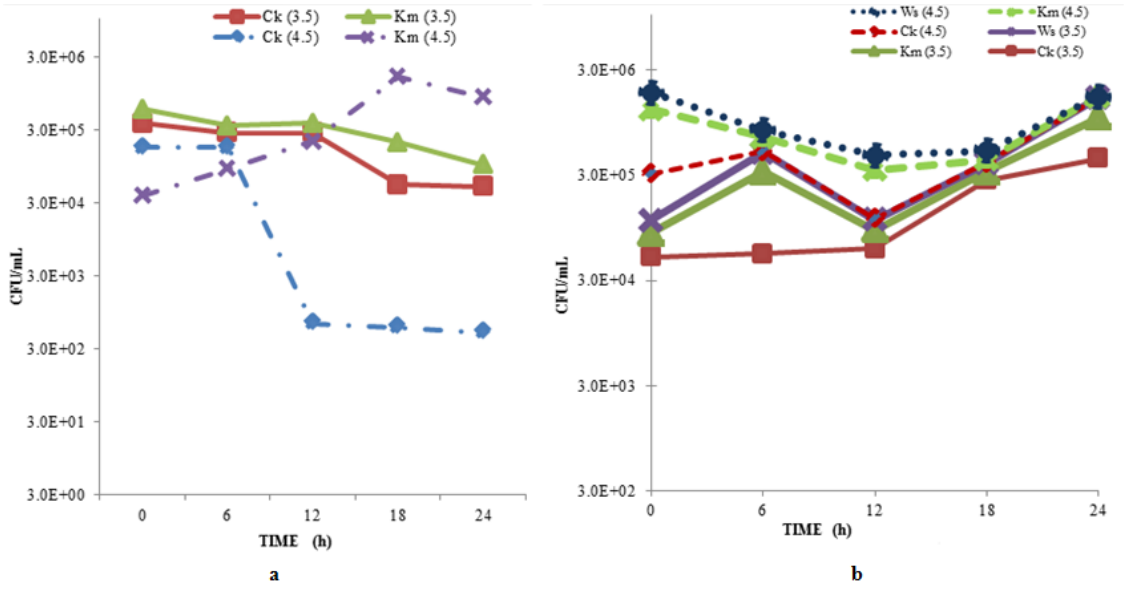

Figure 3 Impact of H_2O_2 and *W. saturnus* (Ws) on the mixed culture (*C. krusei* (Ck) and *K. marxianus* (Km)) in cheese whey at pH (3.5, 4.5) and 28 °C (Shake flask). **a)** With 300 ppm H_2O_2 only **b)** With 300 ppm H_2O_2 and 1 % (v/v) *W. saturnus*

C. krusei was efficiently inhibited in mixed cultures (*C. krusei* and *K. marxianus*) and (*C. krusei*, *K. marxianus* and *W. saturnus*) at pH 4.0, 28 °C and 400 ppm of H_2O_2. Lower CFU/mL of $1.7x10^3$ was observed for *K. marxianus* at pH 4.0 and 28 °C. On the contrary, when *C. krusei* was grown along with *K. marxianus* and *W. saturnus*, *K. marxianus* was observed at high CFU/mL of 2.4×10^3 (as compared to *C. krusei* when grown along with *K. marxianus*). *K. marxianus* showed remarkable growth at pH 5.5 and 40 °C rather than at other pH values, hence pH 4.5-5.5 was ideal for *K. marxianus*.

Higher ranges of H_2O_2 concentrations

After deducing the optimum amount of H_2O_2 used for the complete inhibition of *C. krusei*, similar concentration was applied for industrial scale fermenter broth to eliminate *C. krusei* without affecting the *K. marxianus*. So, 300 ppm of H_2O_2 was the optimum concentration for inhibiting *C. krusei* in the mixed culture in shake flask experiments. When concentration of H_2O_2 was increased from 300 to 800 ppm in the lab scale fermenter broth, no significant inhibition of *C. krusei* was observed at pH 3.5 and 40 °C (Table 2). *K. marxianus* degraded H_2O_2 at pH 3.5, making H_2O_2 ineffective for *C. krusei* inhibition **(Pinheiro *et al.*, 2002)**. In fermented broth, higher concentration (2400 ppm, 3200 ppm, and 4000 ppm) of H_2O_2 was considered at pH 5.0 and 45 °C. Study was conducted for 6 h, as H_2O_2 got degraded into H_2O and O_2 after 6 h (Table 3). A very high concentration 4000 ppm of H_2O_2 finally could kill *C. krusei* completely in the fermented broth. Higher concentration of H_2O_2 was required due to simultaneous degradation of H_2O_2 by catalase action of *K. marxianus* **(Pinheiro *et al.*, 2002)**.

Table 2 Impact of varying concentration of H_2O_2 on the mixed culture in the fermenter broth at pH 3.5 and 40 °C (Shake flask)

	H_2O_2 (ppm)											
	0		300		400		500		600		800	
	Individual Organisms **(Log $_{10}$ CFU/mL) ± Standard Deviation**											
Time (h)	**Ck**	**Km**	**Ck**	**Km**	**Ck**	**Km**	**Ck**	**Km**	**Ck**	**Km**	**Ck**	**Km**
0	7.88±.01	9.04±.02	8.26±.02	8.97±.02	8.42±.02	8.71±.01	7.17±.03	7.67±.01	6.02±.02	6.14±.05	6.17±.11	5.87±.02
3	7.91±.01	8.89±.01	8.34±.02	8.72±.01	8.12±.02	8.76±.01	6.71±.01	6.15±.05	6.47±.10	6.87±.01	5.34±.03	6.49±.02
6	8.18±.04	7.05±.01	8.18±.03	8.69±.01	7.96±.01	7.96±.01	6.85±.01	6.11±.03	6.04±.04	6.95±.04	5.36±.04	6.87±.02
9	7.78±.01	7.79±.01	8.28±.02	8.80±.01	8.18±.03	8.18±.03	6.77±.01	7.28±.03	6.85±.04	7.32±.07	6.04±.02	6.70±.08
12	7.18±.04	8.12±.06	8.80±.02	8.18±.02	8.32±.02	8.32±.02	6.72±.01	6.90±.01	8.25±.13	8.00±.02	6.47±.03	7.47±.02
24	8.40±.02	8.08±.09	8.45±.02	8.45±.03	8.42±.02	8.42±.02	8.04±.13	8.04±.02	8.45±.06	7.41±.07	8.41±.05	7.98±.02

Legend: Ck- *C. krusei*, Km – *K. marxianus*

Table 3 Impact of higher concentrations of H_2O_2 on the mixed culture in the fermenter broth at pH 5.0 and 45 °C (Shake flask)

Time (h)	H_2O_2 (ppm)					
	2400		3200		4000	
	Individual Organisms **(Log $_{10}$ CFU/mL) ± Standard Deviation**					
	Ck	**Km**	**Ck**	**Km**	**Ck**	**Km**
0	6.18±.04	6.50±.02	5.31±.02	6.31±.02	NG	5.18±.05
2	6.31±.01	6.58±.01	6.18±.03	6.42±.01	NG	5.31±.01
4	6.47±.02	6.57±.01	6.31±.01	6.48±.01	NG	5.31±.03
6	6.52±.01	6.81±.01	6.37±.02	6.54±.01	NG	5.39±.01

Legend: NG- No Growth, Ck – *C. krusei*, Km – *K. marxianus*

Inhibition by *S. aromaticum* oil

A study of the mixed culture (*C. krusei* and *K. marxianus*) at pH 3.5 and 28 °C along with various concentrations of clove oil was performed. It was observed that using clove oil concentration 0.5% (v/v) at pH 3.5 and 28 °C is ideal for *C. krusei* inhibition without affecting much the growth of *K. marxianus* ($1.6x10^7$ CFU/mL) in a mixed culture. However when concentration of clove oil was brought down to 0.4% (v/v) and was used in the fermented broth. *C. krusei* was inhibited at 0 h and *K. marxianus* ($1.7x\ 10^7$ CFU/mL) growth was unhampered at 6 h (Table 4). Clove oil 0.4% (v/v) at similar set of pH and temperature used above was ideal for *C. krusei* inhibition in a mixed culture. *Candida* are associated with infections as they form biofilms, *S. aromaticum* extracts worked against biofilm formation and thus, inhibit the growth of *C. krusei* **(Kim and Lee, 2012)**.

Table 4 The inhibition performed by using 0.4% (v/v) of clove oil at pH 3.5, 28°C in fermenter broth (100 mL)

Time (h)	Individual Organisms **(Log $_{10}$ CFU/mL) ± Standard Deviation**	
	Ck	**Km**
0	7.21±.03	6.78±.01
2	NG	6.91±.01
4	NG	7.08±.04
6	NG	7.26±.03

Legend: NG- No Growth Observed, Ck – *C. krusei*, Km – *K. marxinaus*

Inhibition by nisin

After 24 h of incubation the plates were observed and no yeast species were inhibited by nisin.

Inhibition by *W. saturnus*

A primary test was conducted to investigate the interaction between *W. saturnus* and *C. krusei*, along with *K. marxianus.* From the plate technique, it was concluded that *W. saturnus* could inhibit *C. krusei* but not *K. marxianus.* It is necessary to check whether *C. krusei* is an inducer for the production of the killer protein in *W. saturnus* or the latter naturally produces extracellular protein KT4561.

Usage of *W. saturnus* lyophilized powder

A minimum of 156 µg/mL of lyophilized protein in YEPD media is equivalent to 321.9 µg/mL of lyophilized protein in cheese whey needed for the inhibition of *C. krusei* (Table 5). *W. saturnus* did not show any effect below pH 4.5 and it grows well at 25-45 °C. Also *W. saturnus* grow well at pH 3.5 but failed to produce killer protein at the same pH.

Table 5 The inhibition zone created by the minimum concentration of the killer protein along with varying concentration from the lyophilized supernatant from *W. saturnus*

Media	Lyophilized supernatant concentration (mg/mL) of *W. saturnus*	Protein concentration (µg/mL) of killer protein	Inhibition zone formed by killer protein (cms) (Average + Standard Deviation)
Cheese whey	500	321	1.81±.03
	700	475	1.50±.08
Synthetic media (YEPD)	200	156	1.00±.01
	250	158	1.50±.02
	500	168	2.11±.03
	750	176	2.30±.02

Inhibition by synergistic effect of H_2O_2 and lyophilized *W. saturnus*/ supernatant from *W. saturnus*

4000 ppm of H_2O_2 could inhibit the growth of *C. krusei* in the fermented broth (mono-culture) obtained from continuous aerated fermentation (Table 3); and 200 mg/mL (killer protein concentration is 156 mg/L) was the concentration of lyophilized powder needed for the inhibition of *C. krusei* (obtained from Table 5). A synergistic effect of H_2O_2 and lyophilized powder of *W. saturnus* was studied. The set of experiments conducted at pH 5.0 and 40 °C, where 4000 ppm of H_2O_2 and 200 mg/mL of lyophilized *W. saturnus* powder was added. *W. saturnus* was highly effective in killing *C. krusei*, but in these set of experiments; such an inhibition did not occur because *W. saturnus* possesses peroxidase activity, which along with *K. marxianus* degraded H_2O_2 at a much faster rate than *K. marxianus* alone **(Buzzini *et al.*, 2004)**.

2400 ppm of H_2O_2 was considered along with 200 mg/mL of lyophilized supernatant of *W. saturnus* grown in cheese whey at pH 5.0 and 40 °C. At 24 h, cell concentration of *C. krusei* was reduced (Figure 4). By increasing the concentration of lyophilized supernatant of *W. saturnus* to 300 mg/mL, complete inhibition did not take place in a mixed culture. Simultaneously when 2400 ppm H_2O_2 and 400 mg/mL of lyophilized supernatant of *W. saturnus* was applied, H_2O_2 was degraded between 0-6 h because of the catalase-peroxidase enzymatic activity from *K. marxianus* and *W. saturnus*, but lyophilized supernatant of *W. saturnus* showed activity till 24 h. *C. krusei* (CFU/mL) lowered and showed drastic reduction in cell concentration at 24 h, whereas *K. marxianus* ($1.8x10^7$ CFU/mL) remained unaffected.

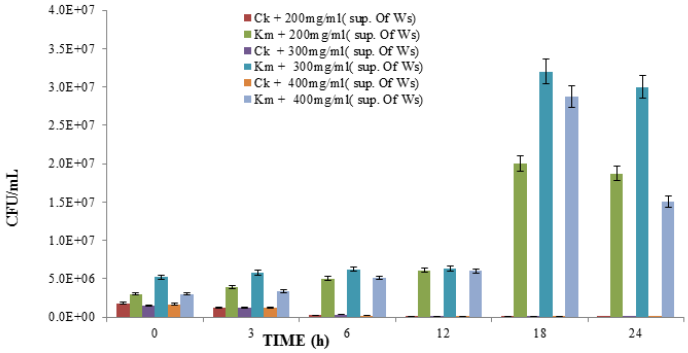

Figure 4 Impact of 2400 ppm H_2O_2 with 200 - 400 mg/mL (156 – 200 µg/mL killer protein) of lyophilized supernatant *W. saturnus* (Ws) powder on the mixed culture (*C. krusei* (Ck) and *K. marxianus* (Km)) in the fermenter broth at pH 5.0 and 40 °C (Shake flask)

Inhibition of *C. krusei* by Ag-KT4561 NPs

Higher concentration of the Ag-KT4561 was observed at 48 h than at 12 h (Figure 5a). Therefore NPs formed at 48 h were considered for this study. A concluding study of the mixed culture (*C. krusei* and *K. marxianus*) along with silver-KT4561 nanoparticles (Ag-KT4561NP) at pH 5.5 and 30 °C showed that 350 µM of Ag-KT461 could efficiently inhibit *C. krusei*. At concentration of 350 µM (Ag-KT4561), the conjugate consists of 1 ppm of reduced Ag.

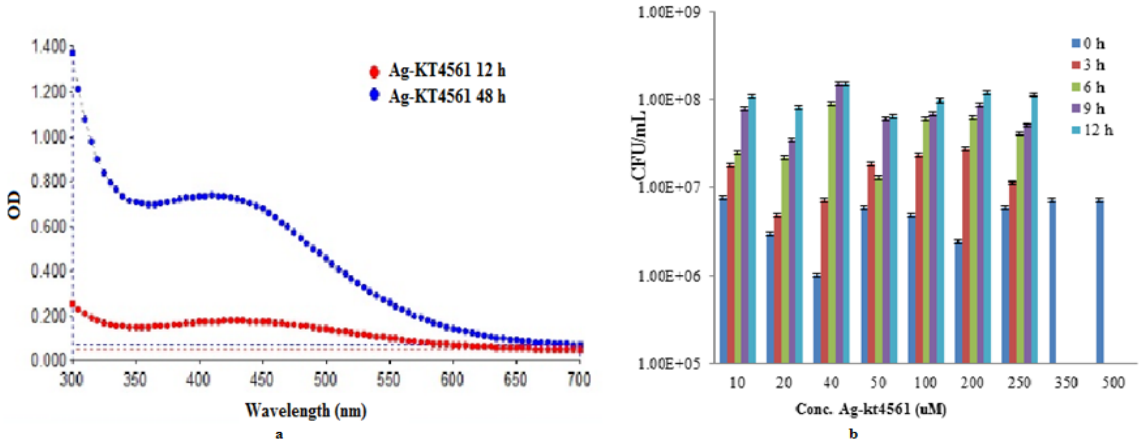

Figure 5 a) UV-VIS spectroscopy showed peak at 410 nm at 12 h and maximum at 48 h during bulk preparation of Ag-KT4561; **b)** A 12 h study of *C. krusei* when various concentrations of Ag-KT4561 was mixed with cheese whey

While growth curves of *K. marxianus* slightly decreases from $8.9x10^8$ to $2.6x10^8$ (Figure 5b). The decrease was less than a log-unit and this might be due to the presence of silver in the Ag-NPs. The other concentrations of the Ag-KT4561 used are as less as 10 µM and as maximum as 1 mM. In any food and feed grade products, a very high concentration of biomolecule based nanoparticle may be toxic for consumption but at a lower concentration of 350 µM (with 1 ppm of reduced silver ions), Ag-KT4561 is an efficient bio-preservative. Another effective approach to use biomolecule based nanoparticle is, no pH adjustment and no temperature adjustment is required. Ag^0 has anti-microbial effects against a wide range of pathogenic microorganisms and since Ag-KT4561 is a combination of killer protein from *W. saturnus* which specifically targets *C. krusei*. A synergistic effect of both (reduced Ag ion and killer protein) can kill *C. krusei* and *K. marxianus* remains partially affected (Figure 5b and 6a). A stability test of Ag-KT4561(350 uM) was performed on cheese whey till 12 h for 20 weeks and every time *C. krusei* was killed after being inoculated at 0 h and *K. marxianus* showed growth at a maximum of $2.3x10^8$ CFU/mL. Though *K. marxianus* growth was affected it did not perish away with the concentration of Ag in Ag-KT4561 (Figure 6b). A tabular representation (Table 6) shows the economics of bio-inhibitor (Ag-KT4561) production in a bulk amount of 20, 000L with a minimum of 1 ppm reduced silver ions.

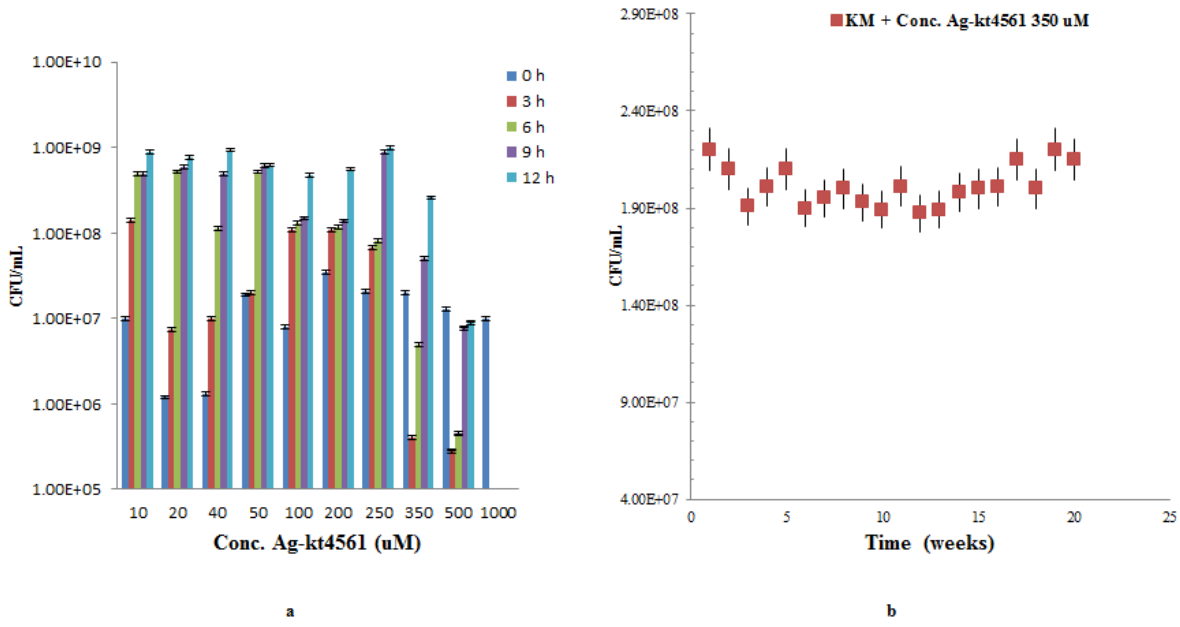

Figure 6 a) A 12 h study of *K. marxianus* when various concentrations of Ag-KT4561 was mixed with cheese whey; **b)** A stability test done for 20 weeks representing growth of *K. marxianus* (KM) at a minimum of 2.1 x 10^8 and no traces of *C. krusei*

Table 6 Bio preservative (Ag-KT4561 conjugate) production of 20,000 L

S. No	Items Required (amount)	Cost of Production (CAD $)
	Reagents and culture medium for stock	
1	Silver nitrate (271.6 g) for 40 L d.H_20	242
2	Culture medium of *W. saturnus* 360 L Preparation cost	306
3	Mechanical stirring of $AgNO_3$ reagent (36W Input power) for 48 h (~ 1.728 kwh)	12.19
4	Centrifugation (700W) for 15 mins (~ 0.175 kwh)	1.23
5	Freeze drying (1200W) for 24 h (~ 28 kwh)	197
	Total	760
6	10% cost of man-power	76

DISCUSSION

The results indicated that 2 M NaCl could effectively inhibit *C. krusei* in a monoculture of *K. marxianus* at temperature 40 °C, pH 3.5 without affecting the growth of *K. marxianus*. However, *C. krusei* showed lower NaCl tolerance than any other yeast species e.g. *Saccharomyces* had different sensitivity towards osmotic stress, but *C. krusei* was inhibited efficiently at 2 M without affecting the growth of *K. marxianus* **(Lynum and Nauth, 2000; Uchida *et al.*, 2005)**. The reported concentration is used to discriminate *K. marxianus* as it is sensitive up to a concentration of 3 M NaCl. Stress-induced by salt induction results into two different phenomena, primarily, ion toxicity and secondly, osmotic stress. Apparently, other physiological changes can also take place such as: a) efflux of intracellular H_2O, i.e. total cell volume deduction; b) transient increase in glycolytic intermediates and finally triggering the hyper osmotic glycerol signaling pathway. Specific species, such as *Saccharomyces* and *Klyuveromyces* can develop systems to counteract to osmotic stress by NaCl. Special features of *Saccharomyces* and *Klyuveromyces* species are that they produce intracellular trehalose under stress conditions to maintain the membrane integrity and stabilizing the proteins **(Kuhn *et al.*, 2004; Wang and Wu, 2008; Davey, 2011)**. However, in large-scale fermenters, it was not possible as it would lead to high utilization of NaCl for the inhibition of *C. krusei*. At industrial scale, such inhibitions performed by utilization of NaCl is difficult not because of the market price which is 16-20 USD per Kg; but the volume of NaCl required was more than 500 Kg for 40 000 L industrial reactor **(Goretti *et al.*, 2009; Kosseva *et al.*, 2009)**. Therefore, NaCl was not a suitable approach for *C. krusei* inhibition.

Apparently, H_2O_2 was found to be effective at 300 ppm when *C. krusei* inhibition was performed at shake flask level. In a shake flask study, it has been shown that 200 ppm could efficiently inhibit *C. krusei* **(Nel *et al.*, 2006)**. Other yeasts, such as *W. saturnus* and *K. marxianus* have shown no inhibition in the presence of H_2O_2 as both the yeasts showed catalase activity. Maximum oxidative stress was observed in case of *Saccharomyces* spp. which was nearly 2-folds more than *K. marxianus* **(Kang *et al.*, 2011)**. Every organism possesses specific antioxidant-defense systems. When *W. saturnus* was added along with *K. marxianus* and *C. krusei*, no inhibition of *C. krusei* was bound to happen as *W. saturnus* even possessed peroxidase activity **(Buzzini *et al.*, 2004)**. Apart from catalase, when *K. marxianus* was introduced to H_2O_2 in the exponential phase, other enzymes, such as superoxide dismutase and glutathione reductase content were even increased to 2-fold **(Nilsson, 2011)**. These were specific antioxidant defensive agents present in *Klyuveromyces* spp. **(Meurman *et al.*, 2007)**.

When pH was brought down from 6.0 to 3.5, it did not affect the inhibition of *C. krusei* either. However, catalase was widely active in a vast range of pH (3.5-10). Apparently, decomposition of peroxidase lowered the pH in the medium **(Pinto *et al.*, 2009; Warnke *et al.*, 2009; Guevara-Flores *et al.*, 2010)**. While varying the pH, temperature was increased to 40 °C and *C. krusei* was efficiently inhibited. When it came to the effect of catalase activity on thermal capacity, 55 °C was the critical temperature and beyond which catalase enzyme was completely destroyed **(Erdei *et al.*, 2011)**. The factors that shifted the physiological process of the glutathione reductases in *K. marxianus* showed higher pH of 6.8 along with temperature (37-40 °C) where it possesses more antioxidant activity **(Ghaly *et al.*, 2005; Pinto *et al.*, 2009)**.

In an aerated continuous fermenter, 4000 ppm of H_2O_2 was required without affecting the growth of *K. marxianus* and *W. saturnus* as both the microorganisms possessed catalase and peroxidase activity which efficiently degraded H_2O_2 during 6 h of inoculation. With such an extreme concentration of 4000 ppm of H_2O_2, *K. marxianus* still had the capacity to resist it. However when fermenter conditions were considered, it was well-stated that 100 ppm of H_2O_2 could cause corrosion of fermenter frame. The other catalysts for corrosion were O_2 and higher temperature **(Sathishkumar *et al.*, 2010)**. Again, •OH, H_2O_2, O_2, H_2, •O_2 could interact with the surroundings; and therefore led to corrosive behavior of many materials including stainless steel **(McMahon *et al.*, 2007; Siddique and Wahid, 2012)**. Though, H_2O_2 might be an inexpensive ingredient for *C. krusei* inhibition, fermenter inner body could have corrosive effects. Therefore, lower concentration of H_2O_2 might be an ideal approach. The use of chemicals depends entirely on the form of free radicals being produced and the damage they may or may not have on the fermenter.

Simultaneously, it was observed that 0.45% (v/v) clove oil could inhibit *C. krusei* without affecting other yeasts. The factor responsible for inhibiting the growth of *C. krusei* was eugenol. Eugenol is the component present in clove oil which can kill *C. krusei* at optimum concentrations **(Noori, 2012)**. *S. aromaticum* (clove oil) 0.4% (v/v) can efficiently bring down the concentration of *C. krusei* in a mono-culture of *K. marxianus*. However, on a large-scale fermentation, it would not be an approachable or economical aspect for inhibition. When the prices were compared, it is observed that 200 Kg of wholesale clove oil will cost USD 12 834.00, and definitely in large scale fermentations, the volume of clove oil required was 160 L, which will not only make the final product oily, but also very expensive. Therefore, it was not an ideal approach for *C. krusei* using clove oil. Similarly when nisin was used for the inhibition as it is one of the biochemical approaches, nisin could not inhibit *C. krusei* at all. Henceforth, the focus was shifted to *W. saturnus* killer protein.

W. saturnus was found to be an effective species which could kill *C. krusei* in a mono-culture of *K. marxianus* without having any implications on *K. marxianus*. Killer protein produced by KT4561 at a concentration of 200 mg/mL (where killer protein concentration is 156 ug/mL) when grown in glucose rich medium;

and 400 mg/mL when grown in a lactose-efficient medium (cheese whey) can inhibit *C. krusei*. The purpose of production of lyophilized supernatant from *W. saturnus* is to justify that *W. saturnus* produces naturally occurring extracellular killer protein KT4561, which strongly inhibited *C. krusei*. The killer protein produced by *W. saturnus* caused cell membrane damage and an independent energy link in between the cell wall receptor and KT4561 at the region of (1→6)-β-D-glucan complex **(Fang *et al*., 2002)**.

More efficient inhibition of *C. krusei* was possible if *W. saturnus* would have been grown in a glucose-rich medium where efficient production of the killer protein could have inhibited *C. krusei*. This study revealed a real understanding of the different microbial species dealt with and different behavioral patterns with respective to the varied inhibitors used for the inhibition of *C. krusei*. The factor to be considered when biochemical approaches such as H_2O_2 were used is whether it again had any effect on the organic matter present in cheese whey. As over the years, approaches have been made to protect the food and humans from consuming it against any oxidative damage. Free radicals such as hydroxyl, peroxyl, and superoxide have been bound to release when biochemical methods are used for inhibiting the food pathogens **(Erdemoglu *et al*., 2007)**.

Killer protein-based nanoparticle showed an effective inhibition for *C. krusei*. It was observed that 350 µM of Ag-KT4561 (with 1 ppm of Ag) could bring in effective inhibition of *C. krusei* within 3 h. But beyond 350 µM concentration could affect the growth *K. marxianus*. *K. marxianus* growth was affected due to the presence of Ag^o but killer protein has no effect on it. Other significant consideration was that no pH or temperature was adjusted, because Ag ion was effective against almost all pathogens. Other benefit of using biomolecule based nanoparticle was that metal nanoparticles were toxic for human or animal consumption but biomolecule based nanoparticles had shown no toxicity so far **(Nel *et al*., 2006; Da Silva *et al*., 2011)**.

CONCLUSION

Biomolecule based nanoparticle approach (Ag-KT4561) for inhibition of *C. krusei* served to be better method than other chemical and biochemical methods used in this study. Other suitable alternative approach might have been ultra-filtration, however, on an industry scale it was an expensive approach. Therefore, Ag-KT4561 was the effective and economic inhibitory approach towards *C. krusei* (non-*Candida albicans* spp.) and it even supported green chemistry. Although the composition of cheese whey was known, further verification and prolonged usage of killer protein-based silver nanoparticle to sustain the antimicrobial effect need to be investigated further.

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